The University of Chicago

THE EFFECTS OF PROTEIN-DEPLETION AND SUBSEQUENT IMMUNIZATION UPON THE RESPONSE OF ANIMALS TO PNEUMOCOCCAL INFECTION

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PATHOLOGY

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By

Robert William Wissler

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THE EFFECTS OF PROTEIN-DEPLETION AND SUBSEQUENT IMMUNIZATION UPON THE RESPONSE OF ANIMALS TO PNEUMOCOCCAL INFECTION*

I. EXPERIMENTS WITH RABBITS

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Evidence that prolonged protein-depletion leads to marked inhibition of the antibody-forming mechanism has been presented in previous reports from this laboratory,[1–5] one of which dealt specifically with depression of the typhoid agglutinin response in hypoproteinemic rabbits.[2] The purpose of the experiments reported in the present paper was to study the response of specifically immunized† protein-depleted rabbits to acute bacterial infection.

The effects of various types of malnutrition upon resistance to infection have been the subject of many investigations.[6–10] In most instances, however, these have been concerned with the effects upon natural resistance and have ignored the possible consequences of poor nutrition upon the immunologic protective mechanisms. Negative or conflicting results have usually been obtained when the relationship of malnutrition to acquired immunity has been studied.[6,11,12]

Although protein is a major structural constituent of antibody and of phagocytic cells, little attention has hitherto been given to the role of protein nutrition and metabolism in affecting the various mechanisms of resistance to infection. Clausen records only two investigations[13,14] in which the effect of low-protein diets upon natural resistance was measured. More recently Sako has presented additional evidence that protein-depletion lowers the native resistance of rats to pneumococcal infection.[15]

The few experiments in which low-protein diets have been found to have no influence upon the capacity of ex-

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† For the purposes of this publication the word immunize will be used to describe the procedure of injecting animals with specific antigenic vaccine and not to denote the production of complete or protective resistance.

1. Cannon, P. R. 1942, J. Immunol. **44**: 107–114.
2. Cannon, P. R., Chase, W. E., and Wissler, R. W. 1943, J. Immunol. **47**: 133–147.
3. Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P. 1944, Ann. Surg. **120**: 514–525.
4. Wissler, R. W., Woolridge, R. L., Steffee, C. H. and Cannon, P. R. 1946, J. Immunol. **52**: 267–279.
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9. Stare, F. J. 1943, Ann. Int. Med. **19**: 735–740.
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11. Zilva, S. S. 1919, Biochem. J. **13**: 172–194.
12. Werkman, C. H. 1923, J. Infect. Dis. **32**: 247–269.
13. Jägerros, B. H. 1902, Skandinav. Arch. f. Physiol. **13**: 375–418.
14. Thomas, E. 1919, Ztschr. Kinderh. **24**: 235–280.
15. Sako, W. S. 1942, J. Pediat. **20**: 475–483.

perimental animals to acquire immunity have been characterized by very short depletion periods[16] or only moderately low levels of dietary protein.[11] Others have recently observed, in agreement with our findings, that the lack of protein, as well as of other constituents in the diet, renders the young rat less able to form agglutinins.[17] In none of these studies was the animal's ability to withstand infection determined following immunization

There appeared to be a need, therefore, for further critical study of the possible relationships between dietary inadequacy, particularly protein lack, and the capacity of specifically immunized animals to resist subsequent infection. Intradermal pneumococcal infection in the rabbit[18,19,20] was chosen for the following reasons: The rabbit possesses little natural resistance to the type 1 pneumococcus and yet can be actively immunized easily to this agent, thereby making it possible to study specific acquired immunity uncomplicated by varying degrees of natural resistance. Furthermore, intradermal infection facilitates administration of a standard infective dose. The superficial nature of the infection simplifies observation of the gross and microscopic progress of the lesion.

The results of the following experiments demonstrate that immunized protein-depleted rabbits exhibit a decreased resistance to intradermal pneumococcal infection and that this results from an impaired immune mechanism, as indicated by the lower antibody titers and higher mortality rates of the protein-depleted animals in comparison with well-nourished controls.

16. Hektoen, L. 1914, J. Infect. Dis. **15**: 279–282.
17. Berry, L. J., Davis, J., and Spies, T. D. 1945, J. Lab. & Clin. Med. **30**: 684–694.
18. Goodner, K. 1928, J. Exper. Med. **48**: 1–20.
19. Goodner, K. 1928, J. Exper. Med. **48**: 413–439.
20. Goodner, K. 1929, J. Immunol. **17**: 279–283.

PROCEDURES AND METHODS

Sixty-four rabbits of both sexes were used of which 27 were adults varying in weight from 1.8 to 2.5 kg at the start of the dietary period and 38 were young animals whose initial weights varied from 560 to 1,240 g. Five experiments were performed, two with adult animals and three with young animals. At the start of each experiment the rabbits were weighed and bled for serum-protein and hemoglobin determinations; they were then divided into two approximately equal groups, comparable with respect to distribution of initial weights and serum-protein concentrations. One group was fed a low-protein diet and the other a stock diet (Alpha Flakes) ad libitum. After a prolonged period of protein-depletion all of the animals, except one in each group, were actively immunized by means of multiple intravenous injections of a formalized and heat-treated penumococcal vaccine. Weight, serum-protein, and hematologic determinations were made both before immunization and at the time of infection which was 6 days after the final injection of vaccine. Following intradermal pneumococcal infection the animals were observed and their lesions measured at frequent intervals. If death occurred the survival time to the nearest half hour was recorded, a blood culture was immediately obtained and an autopsy was performed. In some experiments additional observations were made. For example, in experiments 1, 2, and 3, leucocyte counts, serum-protein and albumin determinations were made both before and 18–21 hours after infection. In experiment 5 leucocyte counts

and rectal temperatures were determined before infection and at frequent intervals thereafter.

Animals and care.—A special strain of rabbits* which for several years has been found to be practically free from common rabbit infections was utilized. The animals were housed in individual wire-bottomed cages and particular care was taken to maintain clean quarters and to keep them isolated from rabbits with snuffles. Attempts to induce protein-depletion in other strains of rabbits have been unsatisfactory because of spontaneous infections.

Rations.—To produce protein-depletion rabbits were fed a ration principally lacking in protein. This consisted of weighed quantities of fresh carrots fed every other day in 100 g portions to adult rabbits and in 200 g amounts to young rabbits. On the alternate days 100 g of a low-protein cookie were fed. This cookie, which except for protein was similar in crude analysis to the control stock diet, had the following ingredients: cornstarch, 77%; lard, 3.25%; "Ruffex," Fisher, 13%; modified Osborne and Mendel salt mixture, 4%; brewer's yeast, 2% and enough oleum percomorphum to furnish 100 I.U. of vitamin A and 12 I.U. of vitamin D per 100 g of diet. The general method of preparing these cookies has been described (2). Carrots contain approximately 1% protein and the synthetic cookies contain approximately 1.5% protein (Nx6.25). Although rabbits offered these diets usually consumed all their carrots, they ate on the average only about 60% of their synthetic diet. On the over-all ration, then, it is estimated that they received an average of only 1 to 2 g of protein daily.

Although it would be advantageous to use a control ration which was similar to this low-protein ration except for higher protein content, our attempts to keep rabbits in optimal nutrition on such a synthetic ration have not been successful. Similar experience with synthetic rations for rabbits has been reported by Hogan and Ritchie.[21]

For these reasons the control animals were fed a commercial rabbit food (Alpha Flakes)† ad libitum, supplemented with approximately 100 g of carrots 2 times per week. Alpha Flakes, according to the manufacturer, has the following proximate analysis: protein, 15%; carbohydrate, 62%; fat, 3.25% and fiber, 12%. Adult animals receiving this diet consumed 75 to 125 g daily, thereby ingesting approximately 15 g of protein. Both young and adult rabbits fed the stock diet gained weight and showed no evidence of malnutrition. Water was supplied ad libitum to all animals.

It should be pointed out that the animals receiving the protein-depletion diet also had a calorie deficit. It is estimated that the control rabbits received approximately 340 calories per day while the rabbits consuming the low-protein diet averaged only 125 to 150 claories per day.

Plasmapheresis.—This technique which was employed in experiment 1 to accelerate protein-depletion has been described.[2] It was used only early in

* These rabbits consist of a mixture of Dutch, American Blue and Common Domestic Stock originally obtained from Dr. C. G. Bull of Johns Hopkins University and hybrid rabbits from The Rockefeller Institute for Medical Research. They are bred exclusively for The University of Chicago at facilities maintained at Lakeside, Michigan.

21. Hogan, A. G., and Ritchie, W. S. 1934, Research Bulletin 219: 1–28, Missouri University Agricultural Experiment Station, Columbia, Missouri.

† Purchased from the Flaked Food Corporation, Racine, Wisconsin.

the depletion period to avoid the risk of killing the animals after they had been weakened by weight loss and anemia.

Serum-protein and blood cell determinations.—Blood was always obtained from a lateral ear vein made hyperemic by applying a small amount of xylene to the tip of the ear. When all of the following determinations were made simultaneously, approximately 5 ml of blood were required. In most instances, however, the albumin and globulin determinations were omitted, making it necessary to obtain only 2 to 3 ml of blood.

Two methods were utilized in determining the serum-protein values, viz., micro-kjeldahl analysis[22] and the densiometric falling-drop method of Barbour and Hamilton.[23] If lipemia is avoided by an adequate fasting of the animals before bleeding, these methods have been found to check consistently within ± 0.2 g% (usually ± 0.1 g%). Albumin and globulin values were determined using the method of Howe.[24] Hemoglobin concentrations were measured by means of the Dick-Stevens photoelectric hemoglobinometer. Leucocyte counts, differential counts, and hematocrit determinations were made using standard methods and equipment.

Infective agent and bacteriologic methods.—The infecting micro-organism was a type 1 pneumococcus which was obtained from the laboratory of Dr. O. H. Robertson. It has a high virulence for laboratory animals and has been utilized in many experimental investigations by Robertson and his colleagues and by others.[25–27] Throughout the period of these experiments the virulence of the organism was maintained by monthly passage through a rabbit and by storage in the cold in a veal infusion broth culture to which 0.2 ml of infected blood had been added. Experimental infection was accomplished in all animals by intradermal injection in the right dorsal flank with 0.10 ml of a 7 to 8 hour culture which was inoculated with two loops of the stock culture and grown in 10 ml serum dextrose veal infusion broth. In non-immunized rabbits this standard infection consistently led to a rapidly spreading cellulitis which resulted in death from septicemia in about 20 hours.

Vaccine.—The vaccine, utilized both for immunization and for agglutinin titration, was formalized and heat-treated as described by Dubos.[28] One lot of vaccine was used throughout. In its undiluted form, as used for immunization, it contained 4.43×10^9 diplococci per ml.

Immunization.—Preliminary experiments demonstrated that two intravenous injections of 1.0 ml quantities of the undiluted vaccine given either 3 or 4 days apart protected most normal adult rabbits against death from a subsequent intradermal infection. In all instances the standard infective dose was given 6 days following the final vaccine injection. This method of immunization and infection was utilized for each of the following experiments with the exception of experiment 3. In this experiment a total of 2.5 ml of vaccine was given in three injections over a 6-day period.

Titration of agglutinins.—Agglutinin titers were always determined from

22. Ma, T. S., and Zuazaga, G. 1942, Indust. & Engin. Chem. **14**: 280–282.
23. Barbour, H. G., and Hamilton, W. F. 1926, J. Biol. Chem. **69**: 625–640.
24. Howe, P. E. 1921, J. Biol. Chem. **49**: 93–107.
25. Robertson, O. H., and Fox, J. P. 1939, J. Exper. Med. **69**: 229–246.
26. Cannon, P. R., and Hartley, G. 1938, Am. J. Path. **14**: 87–100.
27. Lushbaugh, C. C. 1942, J. Immunol. **46**: 151–159.
28. Dubos, R. J. 1938, J. Exper. Med. **67**: 389–398.

serums obtained on the day of infection. Dilutions of these serums in 0.5 ml quantities were prepared beginning at 1:2.5 and continuing by the double-dilution method to 1:320, using 0.86% sodium chloride in distilled water as a diluent. To each tube was added 0.5 ml of a dilute suspension of vaccine (approximately 1:8 dilution of the original). Negative serums obtained before vaccination of the animals were always compared in the same rack with the test serums. After the serum-vaccine mixture had stood for about 1 hour at room temperature, the tubes were centrifuged for 3 minutes at approximately 1,500 RPM and then inspected by gently shaking the tube containing the diluted preinfection serum simultaneously with a tube containing the same animal's negative control serum of the same dilution. The end point was established as the highest dilution showing definite agglutination when shaken sufficiently to re-suspend all the bacteria in the control tube.

Blood culture.—In all experiments except the first, a 0.1 ml sample of the heart blood of each animal was obtained and cultured on a blood agar plate immediately after death

Body temperatures.—In experiment 5 body temperatures were determined before and at frequent intervals after infection by inserting a rectal thermometer for 2 minutes. Care was taken to keep the rabbits quiet during this process to avoid excessive temperature fluctuations.

Histology.—Within an hour after death appropriate sections were taken for histologic study, including in most cases samples of the heart, lungs, liver, kidney, spleen, pancreas, bone marrow and a section of the skin which contained the intradermal lesion. The latter was fixed in Zenker-formol,* imbedded in celloidin, and stained with a modified gram-Weigert stain.[29] The most informative sections were those cut through the deeper layers of the dermis and in a plane parallel to the skin surface. The remainder of the tissues were fixed in Zenker's solution, imbedded in celloidin, and stained with hematoxylin and eosin. Duplicate blocks of liver and heart were fixed in 5% formalin, cut on a freezing microtome and stained with scarlet red.

EXPERIMENTAL RESULTS

In each of the five experiments a greater number of animals fed the stock diet survived the intradermal infection than did those fed the low-protein diet (table 1). Although each experiment was performed with small numbers of animals the experimental procedure was essentially similar throughout, so that it appears reasonable to consider the adult animals as one group and the young animals as a second group. Of the thirteen protein-depleted immunized *adult* animals only one survived whereas six of the ten well-fed immunized control animals survived the same infective dose. Similarly, only two of the fourteen

TABLE 1.—*Mortality data of protein-depleted and normal control rabbits following immunization and infection with type I pneumococcus.*

Exp. No.	Age of rabbits	No. in group	Diet	No. dying	No. surviving
1	adult	5	low protein	4	1
		5	stock	1	4
2	adult	8	low protein	8	0
		5	stock	3	2
3	young	4	low protein	2	2
		5	stock	0	5
4	young	6	low protein	6	0
		9	stock	0	9
5	young	4	low protein	4	0
		5	stock	2	3

* Nine parts of Zenker's solution and one part of neutralized commercial formalin.

29. Wallace, H. M. 1931, Science **74**: 369–370.

young rabbits subjected to a long-continued low-protein diet survived the infection even though they had been immunized identically with their control animals. Of the latter group, seventeen of the nineteen rabbits receiving the stock diet survived. The chi square test reveals that the differences in mortality are highly significant for each age group "(for the 23 adults, $\chi^2 = 7.30$, and P is between 0.05 and 0.01 and for the 33 young animals, $\chi^2 = 15.4$ and P is between 0.01 and 0.001)."

On the other hand when vaccination was omitted as it was for one animal of each diet group in each experiment, an entirely different result was obtained (table 2). Here the well-fed and the protein-depleted rabbits succumbed to the infection at approximately the same time, demonstrating that protein-depletion had produced no appreciable change in the inadequate natural resistance of

TABLE 2.—*Survival times of non-immunized protein-depleted and control rabbits.*

Animal No.	Experiment No.	Diet	Survival time (hrs.)
6	1		21
21	2	low protein	22
32	3		23
45	4		18
Average			21.0
12	1		25
27	2		23
38	3	stock diet	21
55	4		20
64	5		20
Average			21.8

TABLE 3.—*Effect of low-protein diet upon weight, serum-protein, hemoglobin, agglutinin formation and survival to infection with type 1 pneumococcus in adult rabbits.*

Exp. No.	Rabbit No.	Initial data		Diet period	Data at time of vaccination			Data relevant to infection with type 1 pneumococcus		
		Wt. g	Serum-protein g%[b]	Diets and duration[c]	Wt. g	Serum-protein g%[b]	Hgb. g%	Agglu. titer[d]	Hrs. surv.[e]	Blood cult. at death[f]
	1[a]	1850	5.91	Low-	1290	4.90	10.3	20	20.5	—
	2[a]	1920	5.67	protein.	1500	5.21	9.7	20	29.5	—
	3[a]	2000	6.09	105 days	1370	4.00	11.0	20	29.0	—
	4[a]	1850	5.64		1420	4.90	9.8	80	S	—
	5[a]	2350	5.91		1600	5.64	10.7	80	31.0	—
1	Av.	1994	5.84		1436	4.93	10.3	44	—	—
	7	1870	5.80	Stock	2400	5.70	15.0	320	S	—
	8	1970	5.80	diet.	2620	6.23	14.6	40	47.0	—
	9	2240	6.01	105 days	2890	6.12	14.3	80	S	—
	10	2520	5.80		3500	6.36	16.0	80	S	—
	11	2490	6.71		3440	6.67	12.5	80	S	—
	Av.	2218	6.02		2970	6.22	14.5	120	—	—
	13	2070	6.53		1490	5.39	10.4	20	36	pos.
	14	2190	5.50		1410	5.14	8.1	20	41	pos.
	15	2410	5.77	Low-	1720	5.21	9.5	20	59	pos.
	16	2270	6.09	protein.	1280	5.28	7.5	5	28	pos.
	17	2150	5.84	96 days	1260	5.46	9.2	5	41	pos.
	18	2450	6.57		1700	5.60	8.8	20	71	pos.
	19	2260	6.01		1470	5.60	8.6	neg.	35	pos.
	20	2170	6.36		1460	5.14	9.3	20	44	pos.
2	Av.	2246	6.08		1474	5.35	8.9	14	—	—
	22	2100	6.01		2720	6.50	13.5	160	S	—
	23	2190	6.44	Stock	2710	6.57	16.1	40	46	pos.
	24	2320	6.23	diet.	3040	6.20	13.7	20	36	pos.
	25	2170	5.98	96 days	2670	7.16	13.8	40	43	pos.
	26	2160	6.47		2620	6.67	13.1	40	S	—
	Av.	2188	6.23		2752	6.62	14.0	60	—	—

[a] In addition to the low-protein diet plasmapheresis was employed in these animals to assist in protein-depletion. The amount of serum removed varied from 112 to 181 ml.
[b] Micro-kjeldahl determination.
[c] Low-protein diet was composed of 100 gms of carrots alternated daily with 100 gms of synthetic low-protein cookies. The stock diet consisted of "Alpha Flakes" ad libitum supplemented with 100 gms of carrots twice weekly.
[d] Determined with serum obtained on day of infection (6 days following the final dose of vaccine).
[e] S, survived.
[f] —, not determined.

TABLE 4.—*Effect of low-protein diet upon weight, serum-protein, hemoglobin, agglutinin formation and survival to infection with type 1 pneumococcus in young rabbits.*

Exp. No.	Rabbit No.	Initial data		Diet period	Data at time of vaccination			Data relevant to infection with type 1 pneumococcus		
		Wt. g	Serum-protein g%[b]	Diets and duration[c]	Wt. g	Serum-protein g%[b]	Hgb. g%[d]	Agglu. titer[e]	Hrs. surv.[f]	Blood cult. at death
	28	1170	5.74	Low-	1090	4.80	8.6	80	S	—
	29	1160	5.60	protein.	1070	4.59	7.0	40	27	pos.
	30	1080	5.77	117 days	950	4.45	8.0	20	43	pos.
	31	1220	5.11		950	4.62	7.4	80	S	—
3	Av.	1158	5.56		1015	4.62	7.8	55	—	—
	33	1140	4.80		2810	5.50	11.2	320	S	—
	34	890	5.28	Stock	1780	6.64	13.2	320	S	—
	35	940	5.84	diet.	1960	5.77	11.4	320	S	—
	36	1020	4.83	117 days	2490	6.05	13.1	640	S	—
	37	740	5.80		2480	6.01	12.1	640	S	—
	Av.	946	5.30		2304	5.99	12.2	448	—	—
	39	750	5.51		860	5.28	7.7	20	52	pos.
	40	770	5.30	Low-	660	5.21	10.1	neg.	20	pos.
	41	790	5.53	protein.	670	3.51	7.7	20	24	pos.
	42	1050	5.73	156 days	1080	5.28	7.6	neg.	51	pos.
	43	860	5.95		970	5.14	8.0	20	36	pos.
	44[a]	1430	5.74		930	5.28	—	10	31	pos.
4	Av.	942	5.63		862	4.95	—	13	—	—
	46	600	4.78		2930	5.88	13.4	80	S	—
	47	870	5.50		2550	6.61	12.7	160	S	—
	48	980	4.60		2710	6.12	13.9	160	S	—
	49	760	5.49	Stock	2380	6.67	16.2	80	S	—
	50	710	5.99	diet.	2860	6.12	14.4	160	S	—
	51	560	4.93	156 days	2700	6.57	13.9	80	S	—
	52[a]	1410	5.88		3230	6.64	—	320	S	—
	53[a]	740	6.47		2770	5.98	—	160	S	—
	54[a]	1420	4.86		2440	6.53	—	320	S	—
	Av.	894	5.39		2730	6.35	—	169	—	—
	55	680	5.42	Low-	1070	5.18	8.1	20	30	pos.
	56	930	4.83	protein	1170	4.90	8.3	10	45	pos.
	57	1050	4.69	175 days	1420	4.73	7.6	40	47	pos.
	58	810	4.59		1180	3.95	6.3	20	41	pos.
5	Av.	868	4.88		1210	4.69	7.6	23	—	—
	59	810	4.65		3120	7.02	13.7	40	37	pos.
	60	730	5.14	Stock	3040	7.05	13.1	40	35	pos
	61	800	4.76	diet.	3210	6.36	14.0	80	S	—
	62	900	4.24	175 days	3075	6.47	14.5	320	S	—
	63	1060	4.69		3020	6.20	15.0	160	S	—
	Av.	860	4.70		3093	6.62	14.1	128	—	—

[a] Transferred to this group from stock diet one month after the beginning of the diet period. Their diet period was 126 days.
[b] Densiometer determination.
[c] Low-protein diet was composed of 200 gms of carrots alternated daily with 100 gms of synthetic low-protein cookies. The stock diet consisted of "Alpha Flakes" ad libitum supplemented with 100 gms of carrots twice weekly.
[d] —, not determined.
[e] Agglutinin titers determined with serum obtained on the day of infection (6 days following the final dose of vaccine).
[f] S, survived.

the young or adult rabbits.

The pertinent data for each of the immunized animals is summarized in tables 3 and 4. The adult animals fed the low-protein low-calorie ration lost much weight whereas the young animals fed the same ration failed to gain weight. Both age groups consuming this inadequate ration became hypoproteinemic and anemic as contrasted to the comparable animals consuming the stock diet. Of further significance is the fact that the majority of the rabbits fed the low-protein ration consistently showed a diminished acquired immunity following immunization as judged by the agglutinin titer on the day of infection. The correlation was excellent between the concentration of agglutinins and the ultimate resistance as judged by survival, in both the depleted and control animals. Rabbits with a titer of less than 1–80 always developed septicemia and died; whereas those with a titer of 1–80

or above usually survived. Positive blood cultures were obtained from all of the rabbits dying following infection in experiments 2 to 5.

SUPPLEMENTARY OBSERVATIONS

In the course of these experiments other data were obtained relating to the effects of protein-starvation and the general reaction to infection of protein-depleted rabbits as compared to well-nourished controls.

Leucocyte counts.—Leucocyte counts were determined before the diet period was begun and again just before infection in experiment 1, 2 separate counts being made each time. Differential counts were obtained only at the final pre-infection bleeding. The results are shown in table 5. Here it can be seen that there was no significant variation in the total leucocyte counts of the protein-depleted animals as compared to those fed the stock diet. On the other hand, there appeared to be a moderate lymphopenia and an accompanying increase in polymorphonuclear leucocytes in the depleted rabbits' blood after a prolonged low-protein diet period. A similar difference between protein-depleted young animals and their controls is illustrated in table 6 (experiment 4). Statistical analysis demonstrates that the difference between polymorphonuclear leucocytes of the two diet groups is significant in experiment 1, and that the difference in lymphocytes is highly significant in experiment 4.

Of more importance than the concentration of circulating leucocytes in uninfected animals is the influence of infection upon these levels. For this reason an attempt was made to determine the leucocyte response of the two diet groups 20 hours after infection (tables 5 and 6). At least 2 factors prevent evaluation of these results. First, one cannot assume that there was an equal stimulus for leucocytosis in each animal during this 20-hour period since the organisms must multiply at different rates in animals with differing immunity. Second, it is characteristic for animals with a pneumococcal septicemia to develop a leucopenia regardless of their state of nutrition.[18] This phenomenon, which is well illustrated by the tabulated data, makes it difficult to establish the role of protein-depletion in the development of leucopenia by means of a single

TABLE 5.—*The influence of a low-protein diet and infection upon the circulating leucocytes and the differential counts (experiment 1).*

Animal No.	Diet	Initial leucocyte counts		Preinfection leucocyte counts				Leucocyte counts 20 hrs. after infection			Died or survived[b]
		WBC 1000/ cmm	WBC 1000/ cmm	WBC 1000/ cmm	WBC 1000/ cmm	Polys. 1000/ cmm	Lymphs. 1000/ cmm	WBC 1000/ cmm	Polys. 1000/ cmm	Lymphs. 1000/ cmm	
1	Low protein	—[a]	4.50	8.30	6.05	4.23	1.82	0.75	0.18	0.57	D
2		4.80	6.10	6.30	4.45	2.49	1.96	1.05	0.48	0.57	D
3		6.20	6.90	7.00	4.90	3.38	1.52	0.90	0.23	0.67	D
4		6.20	4.40	5.10	2.50	1.35	1.15	7.00	3.36	3.64	S
5		8.10	6.70	—	6.80	3.74	3.06	0.70	0.23	0.47	D
6		6.20	7.90	7.80	4.70	2.58	2.12	0.55	0.04	0.51	D
Av.		6.30	6.08	6.90	4.90	2.96	1.94	—	—	—	
7	Stock	7.00	9.10	7.10	4.30	0.99	3.31	10.35	4.14	6.21	S
8		5.10	5.60	4.70	3.45	1.45	2.00	0.80	0.29	0.51	D
9		5.60	5.20	5.90	4.25	1.36	2.89	5.50	3.47	2.03	S
10		7.20	7.70	6.20	5.35	1.07	4.28	4.55	2.86	1.69	S
11		8.10	4.50	4.80	1.98	0.71	1.27	3.05	2.19	0.86	S
12		6.40	4.50	6.30	3.95	1.66	2.29	0.35	0.23	0.12	D
Av.		6.57	6.10	5.83	3.88	1.21	2.67	—	—	—	

[a] —, not determined.
[b] D, died; S, survived.

leucocyte count subsequent to infection.

It seemed that the bone marrow response might be reflected more adequately by the concentrations of the circulating leucocytes determined at frequent intervals following infection. The changes which occurred in the granulocytes during the first 20 hours after infection in experiment 5 are illustrated graphically in figure 1. The immunized protein-depleted rabbits, all 4 of which failed to survive, showed only slight granulocytic responses following infection and by 10 hours they had developed the granulocytopenia characterizing an overwhelming pneumococcal septicemia. On the other hand the 2 normal rabbits, which were also immunized and failed to survive, responded with a transient leucocytosis followed by a more slowly developing leucopenia. In striking contrast to the response of these groups was the sustained granulocytosis of the 3 surviving vaccinated control rabbits. The average concentrations of lymphocytes in these groups showed no striking changes. In general, they tended to rise or fall slightly with the granulocytes.

TABLE 6.—*The influence of a low-protein diet and subsequent infection upon the circulating leucocytes and the differential counts (experiment 4).*

Animal No.	Preinfection leucocyte counts			Leucocyte counts 20 hrs. after infection			Died or survived[a]
	WBC 1000/ cmm	Polys. 1000/ cmm	Lymphs. 1000/ cmm	WBC 1000/ cmm	Polys. 1000/ cmm	Lymphs. 1000/ cmm	
39	8.55	5.47	3.08	4.55	3.59	0.96	D
40	4.45	2.94	1.51	2.50	0.97	1.53	D
41	4.35	2.69	1.66	0.75	0.45	0.30	D
42	3.75	1.09	2.66	1.75	1.14	0.61	D
43	5.50	2.91	2.59	1.15	0.68	0.47	D
44	7.15	6.00	1.15	1.60	1.09	1.51	D
Av.	5.63	3.52	2.11	—	—	—	
46	7.10	2.12	4.98	11.00	6.16	4.48	S
47	6.60	1.12	5.48	12.25	9.08	3.17	S
48	7.20	4.03	3.17	11.15	5.68	5.47	S
49	7.65	5.65	2.00	12.90	11.48	1.42	S
50	5.40	0.76	4.64	7.45	6.18	1.27	S
51	7.50	1.50	6.00	9.75	4.09	5.66	S
52	7.90	4.42	3.48	1.00	0.80	0.20	S
53	8.20	2.70	5.50	2.85	1.82	1.03	S
54	6.50	2.27	4.23	10.80	8.21	2.59	S
Av.	7.12	2.73	4.39	—	—	—	

[a] D, died, S, survived.

Changes in serum-proteins and their fractions.—An attempt was made to determine the effects of protein-depletion, immunization, and infection upon the levels of the serum-proteins and their fractions. It was found that the difference between the serum-protein concentrations of the two diet groups observed immediately before immunization was due to a lowering of the albumin. The average globulin concentrations were approximately equal. In experiment 2, for example, the average albumin concentrations 3 days before immunization were 3.52 g% for the protein-depleted rabbits and 4.88 g% for the control rabbits, while the globulins were 1.44 g% and 1.50 g% respectively. Similar results have been reported by Weech et al. for the protein-depleted dog.[30]

However, when the average globulin concentrations 6 days following immunization were compared with the preimmunization values it was found that the depleted rabbits of experiment 1 had gained 0.36 g% and the controls 0.48 g%. Similarly, in experiment 2 the hy-

30. Weech, A. A., Goettsch, E., and Reeves, E. B. 1935, J. Exper. Med. **61**: 299–317.

poproteinemic rabbits gained only 0.04 g% in average globulin concentration following immunization while the controls simultaneously gained 0.71 g%. At least a part of this more marked globulin increase shown by the well-nourished animals may be accounted for by a greater production of antibody globulin during the immunization period.

Another significant change in the concentrations of circulating protein took place during the first 20 hours of infection. The more severe infectious process in the depleted rabbits led to a further lowering of the serum protein level while the control animals showed little change (table 7). A similar but less striking shift was observed in experiment 3. In this experiment fractionation of the serum-proteins revealed that albumin and globulin share almost equally in the acute protein loss.

Temperature changes during infection. —In view of the fact that some investigators[31,32,33] have observed that body temperature is important in determining the resistance of animals to pneumococcal infection, the rectal temperatures of the rabbits were determined at 8-hour intervals during infection in experiment 5 (table 8). The average pre-infection temperature was 103 F with no significant difference between the depleted

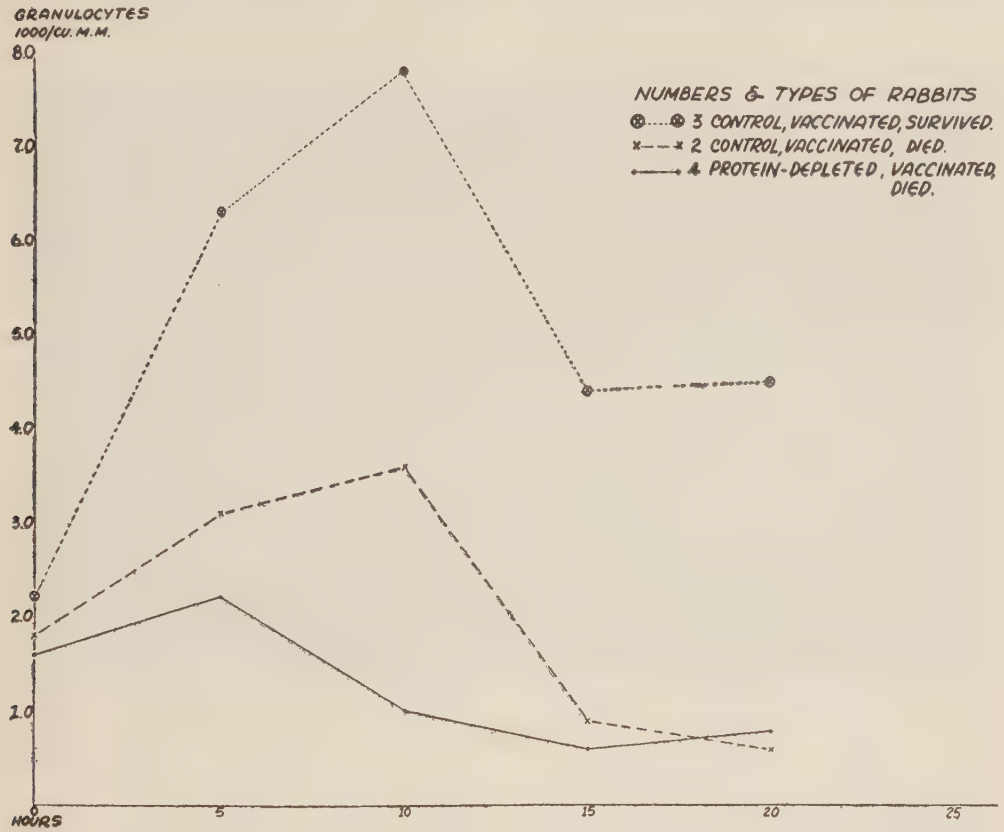

FIG. 1.—Average changes in concentration of circulating granulocytes during infection, experiment 5.

31. Enders, J. F., and Shaffer, M. F. 1936, J. Exper. Med. **64**: 7–18.
32. Strouse, S. 1911, J. Exper. Med. **11**: 743–761.
33. Findlay, G. M. 1923, J. Path. & Bact. **26**: 485–495.

and control animals. Subsequent to infection the control animals developed a greater temperature elevation than the depleted animals and failed to show the marked terminal fall in temperature observed in the low-protein group. Evidence that the elevated temperature would not protect a rabbit from subsequent death by septicemia in the absence of adequate circulating antibody was presented by control animals, numbers 59, 60 and 64, all of which developed temperatures of 105.5 F or above and yet failed to survive. Similarly, 2 depleted rabbits, numbers 56 and 58, developed temperatures over 104 F before they died. Thus, although the infection was more pyrogenic for the well-nourished than for the protein-depleted rabbits this does not appear to have been a major factor determining the outcome of the infection.

TABLE 7.—*Changes in serum-protein concentration during pneumococcus infection in rabbits (experiment 1).*

Animal No.	Serum-protein g%		Difference g%
	At time of infection	Approx. 20 hrs. after infection	
1	5.00	4.00	−1.00
2	4.80	3.88	−0.92
3	5.04	3.29	−1.75
4	5.39	5.04	−0.35[a]
5	5.60	4.48	−1.12
Av.	5.17	4.14	−1.03
7	5.70	5.74	+0.04
8	6.16	5.64	−0.42[b]
9	6.78	7.02	+0.24
10	6.33	6.47	+0.14
11	6.50	6.61	+0.11
A	6.29	6.30	+0.01

[a] Only survivor in this group.
[b] Only animal dying in this group.

General gross and microscopic observations.—The general appearance of a young protein-depleted rabbit and a comparable control is shown in figure 2. The adult depleted animal was similar in appearance except that the skeleton was larger. Loss of hair was often marked. As one might expect, these extremely emaciated animals showed marked depletion of adipose reserves and atrophy of skeletal muscles and internal viscera. Accompanying the hypoproteinemia, ascites and hydrothorax were occasionally observed. The liver was usually small and yellow-tan.

Microscopically, the most consistent finding in the protein-depleted rabbits was a marked large-droplet fatty change in the liver. The small spleen was usually poor in lymphocytes and in many instances the bone marrow showed a marked atrophy of the myeloid elements accompanied by serous atrophy of fat. In the animals dying of septicemia, whether protein-depleted or not, there was a variable degree of small-droplet fatty change in the myocardium.

Gross and microscopic descriptions of the lesions.—Characteristically, the gross appearance of the lesions gave a good index of the rabbits' ability to resist the pneumococcal infection. In the immunized animals which survived, the usual finding was a well-demarcated, round, circumscribed and moderately edematous area which was warm and red and which reached its maximal size about 30 hours after infection. Such lesions usually decreased in size after 40 hours and in most cases healed without ulceration in about 5 days. On the other hand the animals which failed to survive, whether immunized or not,

TABLE 8.—*Body temperatures before and during infection of protein-depleted and control rabbits (experiment 5).*

Animal No.	20 hrs. preinfection (F)	Post-infection temperatures, F				Survival time (hrs.)[b]
		2 hrs.	8 hrs.	17 hrs.	34 hrs.[a]	
55	102.7	102.0	103.6	101.7	—	30
56	103.8	103.2	104.3	103.5	97.4	45
57	102.7	103.5	103.3	103.3	102.1	47
58	102.8	103.2	103.2	104.2	97.7	41
Av.	103.0	103.0	103.7	103.2	—	—
59	103.4	103.5	104.8	106.5	104.2	37
60	102.8	103.7	104.7	106.6	—	S
61	102.8	102.4	104.3	105.4	104.2	S
62	102.9	103.5	104.7	105.1	104.7	35
63	103.7	103.7	104.7	104.7	103.7	S
64	102.7	102.6	104.8	105.5	—	20
Av.	103.1	103.2	104.7	105.6	—	—

[a] —, not determined.
[b] S, survived.

showed lesions similar to those described by Goodner.[18] These consisted of rapidly enlarging, poorly defined and markedly edematous areas which spread swiftly from the point of infection down over the abdomen or back over the hind leg of the animal or over both. These lesions often reached a diameter of 10 marked interstitial growth of pneumococci with no evidence of phagocytosis by the many heterophils present. In some of the immunized animals which failed to survive, a moderate degree of phagocytosis of pneumococci by granulocytes was observed in the older areas of the lesions (site of the inoculation)

FIG. 2.—Typical appearance of young protein-depleted rabbit (no. 55) and control (no. 60) 7 hours after infection.

cm. in 15 hours and sometimes were characterized by marked hemorrhage and necrosis. When an equal degree of humoral immunity was present, the only significant gross difference between the lesions of the depleted and control animals was the more marked edema observed in the former.

Microscopically, lesions were examined only in animals which did not survive the infection. These revealed a marked acute cellulitis which was often focally suppurative. These lesions resembled those described by Rhoads and Goodner.[34] Bacterial stains showed a but sections taken from the edematous edge of the lesion (abdominal wall) invariably revealed fibrin-rich material containing large numbers of pneumococci and practically no leucocytes. Phagocytosis by macrophages could not be demonstrated in any of the lesions. Except for the slight phagocytic activity by the granulocytes, no significant difference existed between lesions taken from immunized animals which did not survive (mostly protein-depleted animals) and lesions from those control animals which had not been immunized.

A comparison of lesions from similarly treated protein-starved and control animals succumbing at approxi-

34. Rhoads, C. P., and Goodner, K. 1931, J. Exper. Med. 54: 41–50.

mately the same time following infection revealed only slight apparent differences. These consisted of a somewhat more marked local leucocyte response and phagocytosis of pneumococci by granulocytes in the animals receiving the stock diet and a definitely more marked local edema in the protein-depleted animals. No difference in maturity of the leucocytes in the lesions could be ascertained.

There was a striking contrast between the lesions from the immunized protein-depleted animals which died and those from similarly infected but adequately immunized rabbits fed the stock diet and sacrificed at intervals following infection approximately equivalent to the survival time of the depleted rabbits.[27]* In the lesions of these control animals extracellular pneumococci were absent except for large clumps of agglutinated organisms within dense foci of polymorphonuclear leucocytes. These leucocytes contained many bacteria. There was only slight adjacent cellulitis, edema or hemorhage.

COMMENT

These experiments demonstrate that the low humoral antibody level which was observed in both young and adult protein-depleted rabbits following active immunization was accompanied by a diminished capacity of these animals to resist an intradermal infection with virulent pneumococci as compared to similarly immunized control rabbits fed a stock diet. The results confirm our previous observations that prolonged protein-deficiency inhibits antibody production in hypoproteinemic rabbits. Of more significance is the fact that antibody formation was depressed sufficiently in protein-depleted animals to lead to death from an infection which well-nourished animals could usually survive. The humoral antibody level was a good index of the rabbit's capacity to resist pneumococcal infection as evidenced by the excellent correlation between the pre-infection serum antibody level and the mortality or survival of the animals.

In addition to this obvious effect of a prolonged low-protein diet period upon acquired resistance, it is evident that this type of malnutrition also influences the reaction of the animal to acute infection in other ways. The fact that the early leucocyte response may be inhibited in the protein-depleted animal, is undoubtedly important. Although this response is difficult to evaluate in animals with varying degrees of infection, the atrophy of bone marrow and lymphatic tissue observed in these rabbits would lead one to expect an impaired leucocyte output. Furthermore, the depressed phagocytic ability of leucocytes from similarly malnourished animals, as reported by others[35,36] may also weaken the defense response to acute infection.

The more sluggish temperature reaction of the protein-depleted rabbit is apparently not the main factor determining survival and its role in the general response of the animal to infection is difficult to evaluate. A similar depression of body temperature reaction in debilitated and undernourished human beings sometimes leads to difficulty in the diagnosis of acute infections.

There can be no doubt that the protein-depleted animals used in these ex-

* Through the courtesy of C. C. Lushbaugh it was possible to study the original sections of the immunized control animals from his experiments and to compare them with similar aged lesions from these experiments.

35. Gellhorn, E., and Dunn, J. O. 1937, J. Nutrition 14: 145–153.
36. Cottingham, E., and Mills, C. A. 1943, J. Immunol. 47: 503–504.

periments had a severe protein-deficiency. Although it is obvious that there was an accompanying calorie lack and possibly some vitamin deficiency in the low-protein ration, from the practical viewpoint the results are none-the-less significant. Since chronic malnutrition in man and other animals is seldom a single deficiency disease these experiments represent a realistic approach to the problem of decreased resistance in undernourished animals showing marked weight loss (or poor growth), hypoproteinemia and anemia.

SUMMARY

1. Young and adult protein-depleted rabbits showed a lowered output of agglutinins to pneumococci following active immunization compared to similarly immunized well-nourished controls of the same age.

2. This lower humoral antibody response was reflected in a decreased resistance to infection so that it appeared to be the main factor determining the survival or death of the animal. Significantly more of the protein-depleted than control rabbits died. The correlation between the serum agglutinin level and ultimate survival or death was excellent.

3. Although there was little or no alteration in the circulating leucocyte concentrations after a long period of low-protein feeding, there was a relative increase in granulocytes accompanied by a comparable decrease in lymphocytes.

4. The protein-depleted animals exhibited a lowered granulocytic response in the early stages following infection.

5. The decline in serum-protein concentration following a prolonged low-protein diet period was confined to the albumin fraction. However, the protein-depleted animals showed a smaller rise in their globulin fraction following immunization than did the control animals.

6. Protein-deficient animals showed a marked fall in serum-protein concentration during infection whereas the control animals showed a slight rise. Albumin and globulin seemed to share almost equally in these fluctuations.

7. The infection caused a more marked temperature rise in the well-nourished controls than in the protein-depleted rabbits. This did not seem to be the factor determining survival since control rabbits with agglutinin levels lower than 1-80 succumbed to the infection even though they developed temperatures of 105.5 F or above.

8. The most marked general gross and microscopic changes which occurred in the protein-depleted rabbits were extreme emaciation accompanied by loss of adipose reserves, loss of hair, muscular and visceral atrophy, occasional ascites and hydrothorax, fatty change in the liver, depletion of lymphoid elements in the spleen, and serous atrophy of the bone marrow.

9. Grossly, the dermal lesion was small, circumscribed and well-delimited in the animals with adequate humoral immunity. It spread rapidly to become large and markedly edematous in the animals with inadequate humoral immunity. Edema of the lesion was more marked in the depleted animals than in controls with equal immunity.

10. Microscopically, dermal lesions from the animals which failed to survive showed marked acute cellulitis with striking interstitial growth of pneumococci and little or no phagocytosis by the many granulocytic leucocytes present. This was in marked contrast to the interstitial agglutination and the effective phagocytosis of pneumococci by leucocytes seen in adequately immunized well-nourished rabbits sacrificed at similar intervals after infection

THE EFFECTS OF PROTEIN-DEPLETION AND SUBSEQUENT IMMUNIZATION UPON THE RESPONSE OF ANIMALS TO PNEUMOCOCCAL INFECTION*

II. EXPERIMENTS WITH MALE ALBINO RATS

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The present paper describes experiments which were designed to ascertain the influence of protein-depletion upon the ability of immunized rats to combat infection. The albino rat has not been widely used for the study of experimental pneumococcal infection,[1] but it has been found to be susceptible to this micro-organism.[1–3] The rat is particularly suitable for nutritional investigation because its dietary requirements are relatively well known and qualitatively resemble those of man. Furthermore, its small size and ready consumption of synthetic rations facilitate experimental procedures. As with the rabbit, antibody formation in the rat has been shown to be influenced by the state of protein nutrition.[4,5]

For these reasons it appeared probable that the study of the influence of protein-depletion upon resistance to pneumococcal infection, begun in rabbits[6] could be continued profitably in rats.

The following experiments were performed in order to ascertain:

1) the influence of the single food constituent, protein, upon resistance to pneumococcal infection; the other ingredients being kept constant in the low-protein and control rations,

2) the effects of varying the infective dose in relation to the weight of the animal,

3) the effects of passive immunization upon resistance to pneumococcal infection in protein deficiency,

4) the nature of the lowered resistance of the protein-deprived animal as revealed by histologic examination of the lesions of depleted and control rats at frequent intervals following infection.

It soon became evident that, in addition to a decrease in acquired resistance to pneumococcal infection, rats fed a low-protein diet also showed a considerable decline in natural resistance. Hence an effort was made to ascertain the character of this depression of native resistance.

PROCEDURES AND METHODS

One hundred and fifty adult male albino rats were used, of which 76 were protein-depleted and 74 were controls. The general experimental plan was similar to that described previously[6] except that the control rations differed from the low-protein rations only by

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4. Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P. 1944, Ann. Surg. **120**: 514–525.
5. Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R. 1946, J. Immunol. **52**: 267–279.
6. Wissler, R. W. 1947, J. Infect. Dis. **80**: 250–263.

substitution of 22% vitamin-test-casein for an equivalent amount of carbohydrate. Alterations in procedure, necessary in some experiments will be described later.

Animals and care.—The rats were purchased from a single source.* Although their initial weights varied from 181 to 370 g animals of the same age and approximately equal weights were used within a single experiment. The rats were kept in groups of from 4 to 7 in large wire-bottomed cages during the experimental periods. Care was taken to sterilize the cages, water bottles, and food dishes used for new groups of rats in order to prevent spread of intercurrent infection.

Rations.—The low-protein ration (3E) and the control ration (3C) have been described.[5] In some experiments, as will be indicated, the animals were shifted to a very low nitrogen diet (4E) and a control ration (4C) during the period of vaccination and infection.

Serum protein and hematologic determinations.—The rats were routinely fasted for 10–12 hours before being bled. Blood for serum protein, hemoglobin and leucocyte estimations was always obtained from a tail vein using a method previously described.[7] Except in the experiments in which serums were obtained for mouse protection tests not more than 0.8 ml was bled from an animal at one time. All serum-protein concentrations were determined using the densiometric method of Barbour and Hamilton,[8] the instrument being checked bi-weekly with the micro-kjeldahl method. Freely flowing blood from the tail vein was drawn directly into appropriate diluting pipettes for estimating hemoglobin and leucocyte levels. The methods used for these determinations have been recorded.[6]

Bacteriologic methods—The microorganism and methods previously described[6] were used in these experiments. Preliminary tests demonstrated that the same standard infective dose given rabbits (0.1 ml of a 7–8 hour serum-dextrose-broth culture injected intradermally in the right flank) led to the death of non-immunized rats by septicemia in approximately 40 hours. This survival time was about twice that of similarly treated control rabbits. This fact suggested that the well-nourished rat, although susceptible to pneumococcus type 1, possessed greater natural resistance to this organism. The standard infective dose was utilized except in certain experiments in which it was doubled for the heavier control animals.

Vaccines.—Four lots of vaccine were employed, containing the following numbers of organisms.

Vaccine Number	No. of diplococci per ml
2	2.58×10^9
3	2.37×10^9
4	2.50×10^9
5	3.33×10^9

These were prepared in approximately 500 ml quantities by the method of Dubos.[9] Each lot of vaccine represented the yield of organisms from 3 L of broth inoculated heavily and grown from 6–12 hours. Care was taken to stop growth before any appreciable numbers of organisms became gram negative.

Immunization.—In preliminary experiments multiple intravenous injections of 1 ml quantities of undiluted vaccine were given on alternate days.

* Sprague-Dawley, Inc., Madison, Wisconsin.

7. Cannon, P. R., Humphreys, E. M., Wissler, R. W., and Frazier, L. E. 1944, J. Clin. Invest. **23**: 601–606.
8. Barbour, H. G., and Hamilton, W. F. 1926, J. Biol. Chem. **69**: 625–640.
9. Dubos, R. J. 1938, J. Exper. Med. **67**: 389–398.

It was found that 2 to 4 injections were required to insure survival of most of the well-nourished control rats when they were infected 6 days following the final injection of vaccine. In the first experiment 4 injections of vaccine were given on alternate days, but this proved to be more than was necessary. Either 2 or 3 injections of vaccine, given on alternate days, subsequently were found to be adequate.

Agglutinin titration.—This was accomplished as in previous experiments.[6] In order to conserve serum half the volumes of all materials were used and the final quantity of fluid in each tube was 0.5 ml. The tests were performed in Kahn serological tubes instead of Wassermann tubes. In general the agglutinin titers observed in rats were lower than in rabbits immunized comparably, and the agglutinated bacteria were more easily dissociated by continued shaking.

Mouse protection tests.—Attempts to test for natural antibodies in the pooled serums from control and from protein-depleted rats were performed utilizing the method described by Sia[10] with suitable modification (table 4). A modification of the method described by Felton[11] was used to test the correlation between agglutinin titers and the mouse protection potency of immune rat serum.

Blood cultures.—Heart blood cultures were obtained immediately post-mortem from all rats dying or sacrificed subsequent to infection, using methods previously described.[6]

Histology.—All animals were autopsied within 30 minutes after death. In experiments 5 and 6 both the depleted and control rats were sacrificed at varying intervals following infection. The lesions were removed, fixed and sectioned in the manner previously described.[6]

EXPERIMENTAL RESULTS

The influence of protein-depletion upon the survival of infected rats was similar to that observed in rabbits (table 1, experiments 1–4). In every experiment a greater number of the immunized depleted rats than control rats succumbed. These data demonstrate that when the quantity of protein is the only variable between the experimental and control rations, there is a marked depression of resistance of the immunized rat to experimental pneumococcal infection. Protein lack in the rat as in the rabbit was characterized by marked weight loss, hypoproteinemia, and anemia (table 1).

The less striking mortality differences between the two diet groups in experiment 1 were probably influenced by two factors. First, the animals were immunized with an unnecessarily large amount of vaccine so that the protein-depleted animals developed more resistance relative to the infective dose of pneumococci. Second, death of 2 of the depleted animals during the period of immunization and cannibalization by the other rats of the group may have increased the survivors' capacity to form antibody.

That the differences in mortality between the two diet groups in experiments 1 and 2 were not due to the smaller dosage of infecting organisms per body weight which the control rats received was demonstrated in experiments 3 and 4. In these the control animals were infected with quantities of pneumococcal broth culture twice those injected into the depleted animals. Nevertheless, under these conditions of approximately equal dosage per body weight, the mortality differences be-

10. Sia, R. H. P. 1928, Proc. Soc. Exper. Biol. and Med. **26**: 284–286.
11. Felton, L. D. 1930, J. Immunol. **19**: 485–509.

tween the two diet groups were still highly significant.

Since rats consuming a low-protein ration tend to show a gradual decline in food consumption, the quantity of ration fed the control groups in experiment 4 was restricted so as to balance the food intakes. Throughout the diet period the average food intake of the control rats was limited to the quantity eaten by the low protein fed animals on the previous day. This led to a reduction in food consumption of the control animals from the usual level of 20 g to a level of 14 g per rat per day in the latter part of the depletion period. The significant difference in mortality between the two diet groups (table 1) demonstrates that voluntary diet restriction, with the resultant decreased intake of essential dietary factors other than protein, was not responsible for the reduced resistance of the depleted rats.

Although in most of the experiments the decreased resistance of the protein-starved rats was associated with a lowered antibody response to immuniza-

TABLE 1.—*Averaged data for each group of rats.*

Exp. No.	No. of rats	Orig. Wt. and S.E. g	Depletion period: Diet	Depletion period: Duration in days	Intravenous immunization	Data at time of infection: Agglu. titer	Wt. and S.E. g	Serum prot. and S.E. g%	Hb. and S.E. g%	Infective dose (7–8 hr. culture) ml	No. dying	No. surviving
1.	6	324 ±7	3E	170	4 ml vaccine #2 per rat	26	166 ±5	4.83 ±0.30	8.8 ±0.9	0.1	2	4
	10	309 ±5	3C	170		48	322 ±12	6.92 ±0.04	14.7 ±0.2	0.1	0	10
2.	10	318 ±8	3E	188	3 ml vaccine #2 per rat	8.5	146 ±6	4.17 ±0.15	7.5 ±0.9	0.1	8	2
	10	310 ±5	3C	188		26.5	355 ±10	6.95 ±0.10	15.7 ±0.3	0.1	1	9
3.[a]	4	237 ±7	3E	130	3 ml vaccine #3 per rat	10	120 ±3	4.62 ±0.07	9.0 ±0.8	0.1	4	0
	5	226 ±11	3C	130		24	306 ±5	6.81 ±0.12	15.1 ±0.3	0.2	0	5
	4	217 ±3	3E	130	Passively Immunized[c]	25	125 ±10	4.69 ±0.44	10.0 ±0.5	0.1	0	4
	4	201 ±10	3C	130		35	286 ±6	7.09 ±0.09	15.9 ±0.2	0.2	0	4
4.	11	246 ±4	3E[b]	185	2 ml vaccine #3 per rat	27	145 ±5	4.71[d] ±0.13	9.9 ±0.6	0.1	6	5
	9	243 ±4	3C[b]	185			292 ±10	6.61[d] ±0.06	15.9 ±0.2	0.2	0	9

[a] Depleted and control animals in these experiments were shifted to ration 4E and 4C respectively at the beginning of the vaccination period.

[b] Consumption of animals receiving control ration (3C) was limited to that of the animals on the low protein ration (3E).

[c] Immunized passively with Lederle anti-pneumococcus rabbit serum (lot#471N 4953). The protein-depleted animals each received 125 units intrvaenously 24 hours prior to infection while the control animals were given 250 units by the same route at the same time.

[d] These determinations were made at the time of the initial dose of vaccine instead of at the time of infection.

tion (tables 1, 3), other factors appeared to contribute to this loss of resistance. In contrast to the rabbit, the non-immunized normal rat possessed considerable natural resistance to the pneumococcus, a part of which was lost during protein-depletion. The average survival-time of the non-immunized control rats in these experiments was 48.5 hours, or over twice that of control rabbits receiving an equal or smaller infective dose (table 2). In contrast, the non-immunized depleted rats showed an average survival time of only 28 hours. As a result of this decreased natural re-

sistance no single agglutinin level represented a "protective titer" (table 3). Control rats with a titer of 1/10 or above always survived whereas only about half of the depleted rats with a titer of 1/20 survived and all with titers below 1/20 died. Thus, more than twice the humoral antibody level was necessary to protect a depleted rat as compared to a control.

TABLE 2.—*Survival times of non-immunized rats.*

Experiment No.	Time of survival (hours)	
	Protein-depleted	Control
1	23.0 26.0	40 45.5
2	25.0	64.5 46.5
3	27.0	36.5[a]
4	35.5 32.0	58.0[a]
Average	28.1	48.5

[a] These control rats were infected with twice the dose of organisms used for the depleted animals of these experiments.

That adequate circulating antibody will serve to offset this lowered natural immunity is demonstrated by the results of experiment 3 (table 1). In this experiment one group of depleted rats was actively immunized, as in the previous series. A second comparable group was passively immunized 24 hours before infection with a commercial antipneumococcal rabbit serum. This serum was given in amounts which produced agglutinin titers approximately equal to those attained in actively immunized control animals in experiment 2. All of the passively immunized depleted rats survived and all of the actively immunized depleted rats died. These results support the concept that the decreased resistance of the actively immunized protein-depleted rat is due in part to a lowering of the natural resistance and in part to a decreased capacity to form specific antibodies. It appeared that the hypoproteinemic rat could withstand the infection if the serum antibody level were equal to that usually found in similarly immunized control animals.

In experiment 4 the actively immunized depleted rats developed an average agglutinin titer slightly greater than that of the control group. In spite of this 6 of the 11 protein-depleted animals succumbed while all of the controls survived (table 1). In the light of the previous experiments these results are difficult to interpret. One might conclude that when the factor of inanition is controlled, as it was in this experiment, protein-deprivation manifests itself in decreased natural resistance rather than depressed antibody production. However, it should be pointed out that equal average antibody levels were observed in the low-protein and control diet groups in two subsequent experiments* in which the diet intakes were not equalized. No final conclusion can be reached, therefore, regarding the signif-

TABLE 3.—*The influence of the agglutinin levels upon survival of actively immunized rats. (experiments 1–4)*

Agglutinin titer	Protein-depleted rats		Control rats	
	No. dying	No. surviving	No. dying	No. surviving
0½	2	0	1	0
5	5	0	0	0
10	10	0	0	7
20	4	5	0	13
40	0	4	0	8
80	0	1	0	5

icance of the identical humoral antibody levels in experiment 4. Unpublished experiments using 2 large series of rats with balanced dietary intakes have shown a marked depression in

* The survival rates were not determined in these experiments because the rats were sacrificed for histologic examination of the lesions in one and bled for mouse-protection studies in the other.

hemolysin formation in the protein-depleted rats compared to the inanition controls.

Mouse-protection studies.—In order (1) to investigate the mechanism concerned in the decreased natural resistance of the protein-depleted rats, and (2) to study the relationship between agglutinin titers and the protective potency of immune rat serums, suitable mouse-protection studies were performed. In the tests for natural antibody the general plan was to pool equal samples of serum from each animal of a group of nonimmunized depleted rats and from each of a group of nonimmunized control rats, similar to those used in the preceding experiments. Equal amounts of the two undiluted pooled serums were injected intraperitoneally into mice. Later these mice, as well as mice not receiving serums, were injected intraperitoneally with varying dilutions of a 7–8 hour culture of pneumococcus. No appreciable protective action for either type of serum could be demonstrated by these tests (table 4). The results suggest, therefore, that the natural resistance of the non-immunized rat to pneumococcal infection is not dependent upon the presence of normal circulating antibody similar to that demonstrated by these techniques in the serums of more resistant species.[10,12,13] The loss of the natural resistance in the depleted rat is, therefore, not due to a decline in concentration of natural humoral antibody.

TABLE 4.—*End points of mouse-protection tests performed with pooled serums from non-immunized protein-depleted and control rats.*

Groups of mice	Test #1[a] ml of culture	Test #2[b] ml of culture	Test #3[c] ml of culture
No serum	1.0×10^{-9}	—	0.5×10^{-9}
Non-immunized depleted serum	—	1.0×10^{-5}	0.5×10^{-9}
Non-immunized control serum	1.0×10^{-9}	1.0×10^{-5}	0.5×10^{-8}

[a] Two mice were used for each dilution of the culture which was injected in 1.0 ml quantities intraperitoneally 4 hours following the injection of 1 ml of the undiluted pooled serum in the same site.

[b] Same procedure as in Test #1 except that 0.7 ml quantities of pooled depleted and control rat sera were used. The lower endpoint of the test was due to poor growth of the culture.

[c] Same procedure as for Test #1 except that 0.5 ml quantities of the appropriate sera were used and 0.5 ml quantities of each culture dilution were injected 1.5 hours later.

Other mouse-protection tests were done to determine whether the agglutinin titers gave an accurate index of the protective potency of immune rat serum. These were performed by injecting a series of mice each with 0.5 ml of varying dilutions of pooled serums from actively immunized protein-depleted rats using two mice for each dilution. Another group of mice was injected similarly with pooled serums from immunized control rats. Subsequently each mouse was infected with 0.5 ml of a 10^{-6} dilution of an actively growing culture. The end-point of the mouse-protection test correlated well with the end-point of the agglutinin titration although the former was usually one double dilution higher than the latter.

Histologic study of the lesions.—To gain a clearer understanding of the defense mechanisms operative in the protein-depleted and control rats, lesions were examined microscopically at varying intervals after infection in 4 groups of animals, viz., protein-depleted non-immunized, control non-immunized, protein-depleted immunized and control immunized rats (table 5). A single animal of each group was sacrificed at 2.5, 5, 10, 15, 20 and 27.5 hours after infection. Careful evaluation of sections from these lesions stained with the gram-Weigert technique revealed the following characteristics for each group.

1. Protein-depleted non-immunized rats. This group exhibited remarkably

12. Kelley, W. H. 1932, J. Exper. Med. **55**: 877–888.

13. Bull, C. G., and McKee, C. M. 1921, Am. J. Hyg. **1**: 284–300.

little histologic evidence of resistance. In the earlier periods of the infection (2.5 and 5 hours) there was practically no evidence of phagocytosis by polymorphonuclear leucocytes, although it was estimated that these cells were present in only slightly smaller numbers than in the control groups. The presence of edema was prominent and many pneumococci were disseminated through the edema fluid. By 10 hours enormous numbers of extracellular bacteria were present in all areas of the section and phagocytosis was still slight (Fig. 1). In the later lesions the more peripheral parts consisted of edema fluid containing many organisms but with only a few leucocytes (Fig. 2). Although slight to moderate phagocytosis by polymorphonuclear leucocytes was seen in the older parts of the lesions after 15 hours, little or none could be demonstrated near the borders.

2. Control non-immunized rats. In contrast to the first group, these lesions exhibited much more marked phagocytic activity in the early stages of the infection (Fig. 3) as well as in the later lesions. However phagocytosis was never completely effective so that extracellular organisms were always seen, especially near the edges of the lesions. In the later stages free pneumococci were still apparent forecasting the ultimate inadequacy of the defense (Fig. 4). Lymphocytes and young macrophages were apparent in the 5 hour lesions and were the most prominent cells in the peripheral areas of the lesions after 10 hours, in contrast to their relative scarcity in the lesions from depleted rats. However, there was little evidence

TABLE 5.—*Averaged data for groups of rats sacrificed for histologic examination of lesions (experiment 6).*

No. of rats	Ave orig. wt. and S.E. g	Depletion period		Intravenous immunization	Data at time of infection			Infective dose ml 7–8 hour culture
		Diet	Duration in days		Wt. and S.E. g	Serum prot. and S.E. g%	Hb.[a] and S.E. g%	
7	223 ±4	3E	113	Not immunized	131 ±3	3.69 ±0.11	10.5 ±0.7	0.1
6	201 ±7	3C	113		303 ±5	6.42 ±0.05	15.5 ±0.2	0.1
6	221 ±6	3E	113	2 ml vaccine #5 per rat	120 ±2	3.94 ±0.24	10.7 ±0.4	0.1
6	232 ±13	3C	113		303 ±12	6.46 ±0.19	15.8 ±0.3	0.1

[a] These determinations were made at the time of the initial dose of vaccine instead of at the time of infection.

PLATE 1

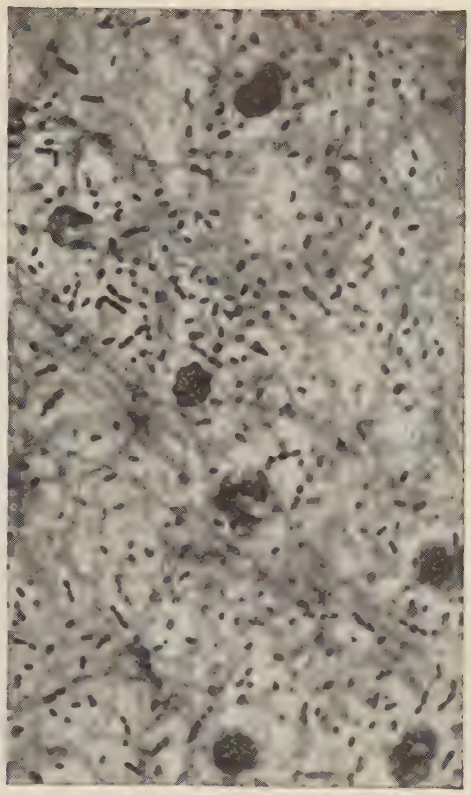
FIG. 1.—Protein-depleted, non-immunized rat. Dermal lesion 10 hours after infection. Note many extracellular pneumococci dispersed through the edematous tissue. Moderate numbers of heterophils. Phagocytosis minimal. (×900)

FIG. 2.—Protein-depleted, non-immunized rat. Edge of dermal lesion 20 hours after infection. Note the numerous extracellular pneumococci diffusely distributed through the fibrin rich exudate with no demonstrable phagocytosis and few leucocytes. (×900)

FIG. 3.—Well-nourished control, non-immunized rat. Dermal lesion 5 hours after infection. Note definite phagocytosis by heterophils but extracellular pneumococci still prominent. (×900).

FIG. 4.—Well-nourished, non-immunized rat. Near center of dermal lesion 20 hours after infection. Despite phagocytosis there are still many extracellular pneumococci. (×900)

1



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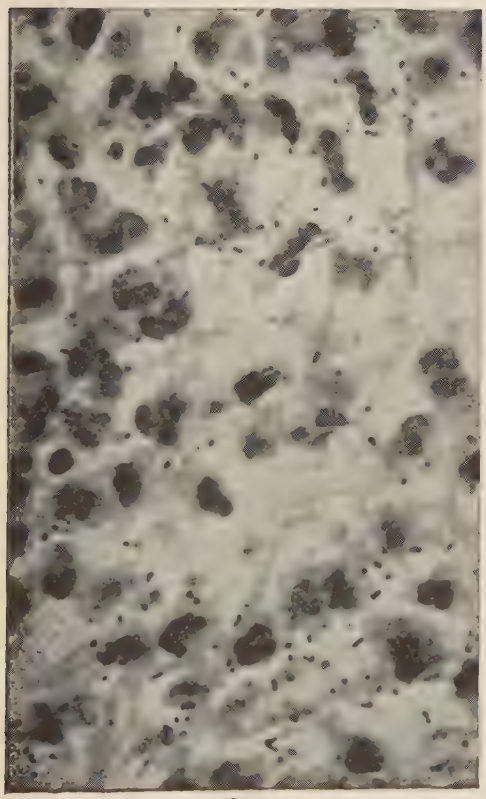

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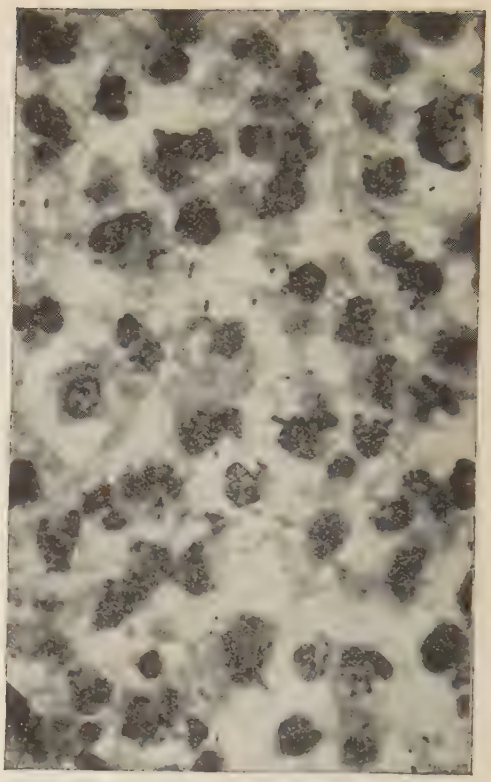

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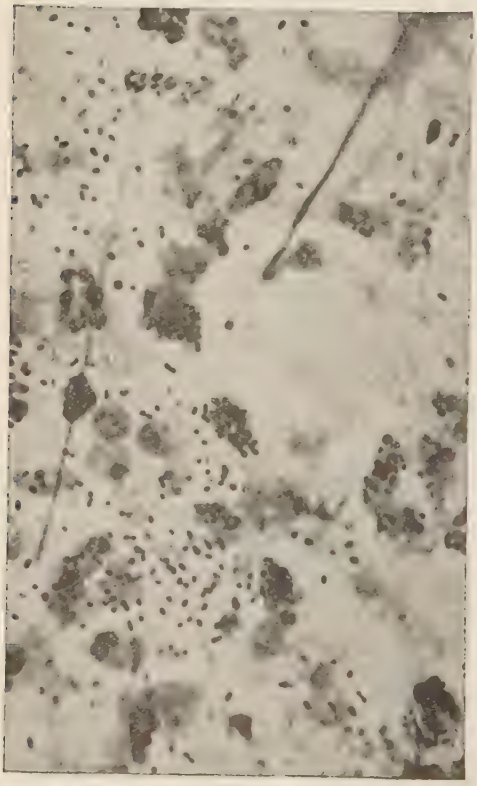

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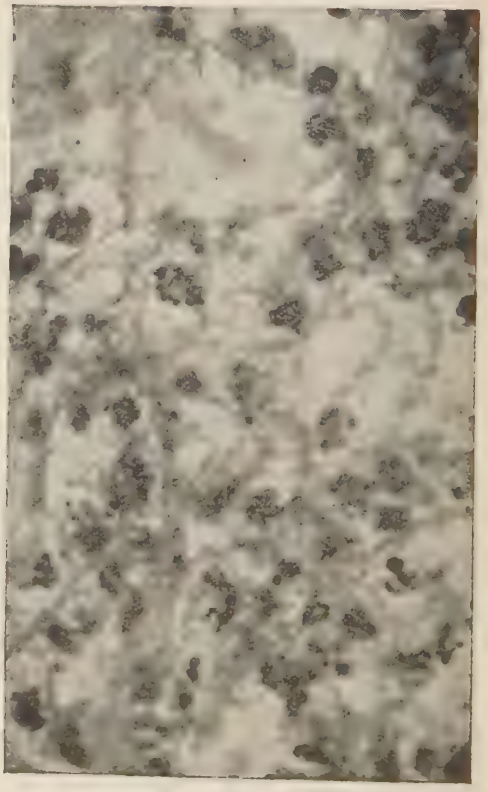

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FIG. 9.—Typical Appearance of Immunized Control Rat 19 Hours after Infection. Note the Edematous Border of the Small Localized Lesion.

of phagocytic activity by these cells at any time. Focal suppuration was seen near the centers of the older lesions.

3. Protein-depleted immunized rats. The lesions from this group were quite different at all stages from those seen in protein-depleted non-immunized rats and resembled more closely those of the control non-immunized rats. Phagocytosis of pneumococci by granulocytes, while only slight at 2.5 hours, was quite prominent at 5 hours and thereafter (Fig. 5). The organisms remained somewhat localized and the lesions showed central suppuration. This localization was imperfect, however, as evidenced by the presence of moderate numbers of extracellular organisms at the edge of the lesions (Fig. 6) in all except one of the animals (27.5 hours). There was slight phagocytic activity by the fixed-tissue-macrophages, best seen in the 5 hour lesion.

4. Control immunized rats. As might

PLATE 2

FIG. 5.—Protein-depleted, immunized rat. Dermal lesion 5 hours after infection. Note the ineffectiveness of phagocytosis and large numbers of pneumococci. (×900)

FIG. 6.—Protein-depleted, immunized rat. Near center of dermal lesion 20 hours after infection. Numerous extracellular pneumococci are seen in spite of large numbers of heterophils present in the exudate. (×900)

FIG. 7.—Well-nourished immunized rat. Dermal lesion 2.5 hours after infection demonstrating active phagocytosis even at this early stage.

FIG. 8.—Well-nourished, immunized rat. Dermal lesion 15 hours after infection. Note the almost complete absence of extracellular pneumococci and the effective phagocytosis.

be expected from their capacity to survive these animals' lesions showed the most effective defense reaction. This was the only group in which phagocytic activity was prominent at 2.5 hours (Fig. 7). At this early stage macrophages as well as polymorphonuclear leucocytes were actively ingesting organisms. Extracellular bacteria were absent at 5 hours and thereafter and the lesion remained localized and tended to suppurate (Fig. 8). Although adjacent edema was prominent, the fluid appeared to be sterile. Polyblast formation was quite marked in the edges of the lesion after 5 hours. The phagocytic effectiveness of these cells could not be assayed, however, as no appreciable numbers of bacteria were ever seen near the borders of the lesions.

In general, the impressions gained from histologic examination of the lesions correlated well with resistance of corresponding groups of rats, as judged by survival. The degree of resistance appeared to depend upon the time of onset and the effectiveness of phagocytosis by polymorphonuclear leucocytes and, to a lesser extent, by histiocytes. In contrast to the rabbit, the non-immunized rat possesses a significant, but not a protective phagocytic activity for pneumococci. A marked reduction in the speed and effectiveness of phagocytic activity occurs during protein-depletion. The humoral immunity developed during immunization appears to improve the function of this depressed phagocytic mechanism to a limited extent. Nevertheless, most protein-depleted immunized rats still have an imperfect ability to localize the pneu-

FIG. 10.—Typical Appearance of Immunized Protein-Depleted Rat 19 Hours after Infection. Note the Marked Edema and Contracture of the Hind Leg Due to the Spread of the Infection into This Area.

mococcus. Only the well-nourished immunized rat is capable of effectively controlling the infection by speedily mobilizing the phagocytic function of both the granulocytic leucocyte and the macrophage.

Supplementary observations.—The blood leucocyte responses of the protein-depleted and the control rats were investigated by means of consecutive counts during the infection (experiment 1). The average circulating levels of leucocytes, lymphocytes and granulocytes were compared in those immunized protein-depleted and control rats which survived. It was assumed that the infective stimulus for leucocytic response would be approximately equal in these two groups. The results of this comparison (table 6) showed that, as with the rabbit, protein-starvation leads to a marked inhibition of leucocytosis. This was reflected in the absence of the characteristic swift rise in granulocytes during the early period of infection in the hypoproteinemic group. Concurrently, these animals showed a slight fall in their lymphocyte levels as contrasted to the progressive rise in the well-nourished rat. There was no significant difference in the leucocyte levels of these two groups before infection.

The typical appearance of a protein-depleted and a control rat are shown in figures 9 and 10. The animals which had consumed a low-protein diet for long periods showed marked emaciation, frequent loss of hair and occasional ascites and hydrothorax. Subcutaneous edema of the thorax between the fore feet was sometimes observed. Grossly, the contrast of the dermal lesions between animals of differing degrees of immunity was not so great as in the rabbit, due to a failure of the rat's skin to become hyperemic over the lesions. Hence palpation gave a better index of lesion size.

COMMENT

Interpretation of these experimental results is complicated by the simultaneous variation of two elements—the natural resistance and the acquired resistance of the rat to the pneumococcus. The results give definite evidence that natural resistance as well as the capacity to fabricate antibodies were depressed in many of the protein-depleted rats. However, it is difficult to interpret the part played by each of tl ese variables quantitatively. This is in contrast to the experiments in rabbits whose native resistance to pneumococcus was negligible even before protein-depletion.

TABLE 6.—*Average white blood cell counts of surviving immunized depleted and control rats at intervals during infection. (experiment 1.)*

Diet	No. of rats	Cell type	White counts (1,000/cmm)			
			Prein-fection	15 hours	28 hours	48 hours
Low protein	5	Total Leucocytes	7.84	8.72	8.65	10.40
		Lymphocytes	5.81	6.21	4.58	6.16
		Granulocytes	2.03	2.72	4.07	4.23
Control	10	Total Leucocytes	7.77	14.91	12.44	13.75
		Lymphocytes	4.70	6.57	7.39	9.62
		Granulocytes	3.07	8.33	5.03	4.12

The fact that natural resistance was reduced in the protein-starved rat amply confirms the observations of Sako.[2] Furthermore, the inhibited phagocytic activity demonstrated histologically in the lesions from non-immunized protein-depleted rats agrees with depressed phagocytosis shown in vitro by leucocytes from rats deprived of protein.[14] The mechanism by which the phagocytic function is impaired needs additional study.

The relative importance of the lower blood leucocyte levels in the infected hypoproteinemic rat is also a matter for

14. Cottingham, E., and Mills, C. A. 1943, J. Immunol. 47: 503–504.

further investigation. In this acute infection the depression of leucocytosis in the protein-starved animal does not appear to be the main factor determining survival since the observations were made with infected animals which ultimately survived. Failure of leucocytosis might be expected to exert a much greater effect in a chronic suppurative process, with its constant drain upon the leucocyte supply. It is of interest that uninfected adult rats (pre-infection counts, table 6) subjected to protein-deficiency in the presence of adequate quantities of other food constituents failed to show the leucopenia observed by others in the young rat fed a diet deficient in several necessary elements, including protein.[15]

Although the contrast between agglutinin levels of the two diet groups was neither as striking nor consistent as in the experiments with rabbits (part 1), there was still a definite contrast in the average titers in three experiments. Several factors prevent the satisfactory estimation of acquired immunity by means of this test. For example, the technique does not measure the total circulating antibody, which with equal titers would be considerably greater in the controls because of their larger plasma volume.[16] Furthermore, it offers no adequate measure of the concentration of tissue antibody which must be an important factor in the outcome of a dermal infection of this type.

There is an apparent inconsistency between the agglutinin titers induced by passive immunization which protected the depleted rats in experiment 3, and the approximately equivalent agglutinin titers resulting from active immunity in experiment 4, in which most of the depleted immunized rats succumbed. This may be the result of some unmeasured variable between the 2 experiments. Perhaps, however, immune rabbit serum of a given agglutinin titer has a higher protective potency than an equivalent titer rat serum. It should be pointed out, also, that the low levels of pneumococcal agglutinins developed by the rat make comparative study difficult. Others, utilizing different microorganisms, have demonstrated much more striking contrasts in agglutinin formation between rats deprived of protein and their controls.[15,17]

From the practical standpoint the important fact is that protein-depletion in the rat appears to exert the double effect of diminishing both natural and acquired resistance to the pneumococcus.

SUMMARY

In general, immunized protein-depleted rats showed a lower agglutinin response and a lower survival rate following infection than did rats fed the control rations. These results are in agreement with those obtained with protein-depleted rabbits.

Administration of the infective dose of organisms on a body weight basis did not significantly alter the survival rates.

Prolonged restriction of the daily dietary intakes of the control rats to the consumption levels of rats eating the low-protein ration had no marked influence upon the relative mortality of the two diet groups. Under these conditions, however, agglutinin response was approximately equal.

In contrast to the rabbit, the normal non-immunized rat possessed a moderate natural resistance to pneumococcal infection. A large part of this resistance

15. Berry, L. J., Davis, J., and Spies, T. D. 1945, J. Lab. & Clin. Med. **30**: 684–694.
16. Benditt, E. P., Straube, R. L., and Humphreys, E. M. 1946, Proc. Soc. Exper. Biol. and Med. **62**: 189–192.
17. Bhattacharya, B. K., and De, S. P. 1943, Ann. Biochem. & Exper. Med. **3**: 137–140.

was lost, however, during protein-depletion.

Adequate passive immunization effectively counteracted the depressed natural resistance of protein-depleted rats, even when the resulting agglutinin levels were no higher than those ordinarily obtained by active immunization of control rats.

Mouse-protection studies, utilizing pooled serums from both non-immunized depleted and control rats, demonstrated no circulating natural antibody which might explain the native resistance of the normal rat or the decrease in this resistance in the protein-depleted rat.

Mouse-protection tests with pooled sera from immunized rats revealed a good correlation between the agglutinin level and the protective potency of immune rat serum.

Histologic examination of lesions of protein-depleted and control rats, immunized and non-immunized revealed marked differences in phagocytic activity among the 4 groups. The polymorphonuclear leucocytes of the non-immunized control rat possessed a considerable, but non-protective, phagocytic activity. Much of this activity was lost as a result of protein-depletion. Immunization of protein-depleted rats, however, produced a marked increase in the phagocytic action of the leucocytes and, to a lesser degree of the macrophages, but extracellular organisms still remained. Similar immunization of the normal rat led to an effective defense against the pneumococci characterized by accelerated phagocytic activity by both granulocytes and macrophages. In these control immunized rats the lesions remained localized and extracellular bacteria were not seen later than 2.5 hours after infection. These observations indicate that the degree of natural and acquired resistance of the rat is related to the effectiveness of phagocytosis. However, they do not fully explain the mechanism by which the phagocytic activity and the natural resistance of the non-immunized protein-depleted rat was reduced.

The blood leucocytosis typical of an acute pneumococcal infection in surviving control rats was relatively slight in the protein-depleted rats. Both granulocytes and lymphocytes were affected It is likely that a part of the decreased resistance of depleted animals is due to an impaired fabrication of leuoccytes.